EFFECT OF SUBSTRATE CONCENTRATION AND pH ON SUCCINIC ACID PRODUCTION

NIK NOR AZIATI BT ABD AZIZ

A thesis submitted in fulfillment
of the requirements for the award of the degree of
Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical & Natural Resources Engineering
Universiti Malaysia Pahang

APRIL 2010

ABSTRACT

The purpose of this research is to study the effect of substrate concentration and pH on succinic acid production. Succinic acid has been produced from the fermentation process by using *Escherichia coli b-strain*. The experiments were conducted in Modified *Schott* Bottle in incubator shaker to maintain anaerobic condition, temperature and agitation speed. Fermentation were carried out at the optimum temperature of *E.coli* growth which is $37^{O}C$ for 72 hours. The results of HPLC analysis showed that increase in fermentation time will increase succinic acid production. The maximum production of succinic acid is at pH 6.0. Initial substrate concentration (5 to 15 g/L) will increase Succinic acid production, but after 15 g/L substrate concentration, the succinic acid production will decrease. From this study, it was observed that the substrate concentration and pH are important factor that affects both growth and growth-associated productions of molecules to increased succinic acid production.

ABSTRAK

Kajian ini dilakukan bertujuan untuk mengkaji kesan kepekatan substrat dan pH terhadap penghasilan asid suksinik. Asid suksinik dihasilkan melalui proses fermentasi dengan menggunakan *Escherichia coli b-strain*. Eksperimen ini dilaksanakan di dalam *schott* botol yang telah diubah suai dan dijalankan di dalam incubator untuk mengekalkan keadaan anaerobik, suhu dan juga halaju adukkan. Fermentasi dilakukan pada suhu optimum bagi pertumbuhan *E.coli* iaitu $37^{o}C$ selama 72 jam. Keputusan eksperimen menunjukkan, peningkatan masa fermentasi akan meningkatkan penghasilan asid suksinik. Penghasilan asid suksinik maksimum adalah pada pH 6.0. Penghasilan asid suksinik meningkat pada kepekatan substrat 5 hingga 15 g/L dan berkurang penghasilannya selepas 15 g/L kepekatan substrat. Hasil daripada analisa menggunakan HPLC menunjukkan kepekatan substrat dan pH merupakan faktor penting dalam pertumbuhan *E coli* bagi penghasilan asid suksinik.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS/ABBREVIATIONS

ATP	= adenosine 5’-triphosphate
C	= carbon
Ca^{2+}	= ion calcium
g	= gram
hr	= hour
H_2O_2	= hydrogen peroxide
kDa	= kilo Dalton
ml	= mililitre
Mn^{2+} and Mn^{3+}	= ion manganese
N	= nitrogen
NAD^+	= nicotinamide adenine dinucleotide
OD	= optical density
S^+	= ion sulphur
µm	= micrometer
^oC	= degree Celcius

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Introduction

Succinic acid, also known as amber acid or butanedioic acid, is a dicarboxylic acid having the molecular formula of $C_4H_6O_4$. After its first purification of succinic acid from amber by Georgius Agricola in 1546, it has been produced by microbial fermentation for the use in agricultural, food and pharmaceutical industries (Menzel *et al.*, 1999).

Glycerol is very competitive with sugars used in the production of chemicals and fuels via microbial fermentation. Glycerol give the highly reduced nature of carbon atoms in glycerol, additional advantages can be realized by using glycerol instead of sugars. Fermentative metabolism would then enable higher yield of fuels and reduced chemicals from glycerol compared with those obtained from common sugars such as glucose or xylose. Anaerobic fermentation is also preferred because of its lower capital and operational costs when compared to aerobic fermentations (anaerobic fermenters are less expensive to build and operate than their aerobic counterparts and also use less energy).

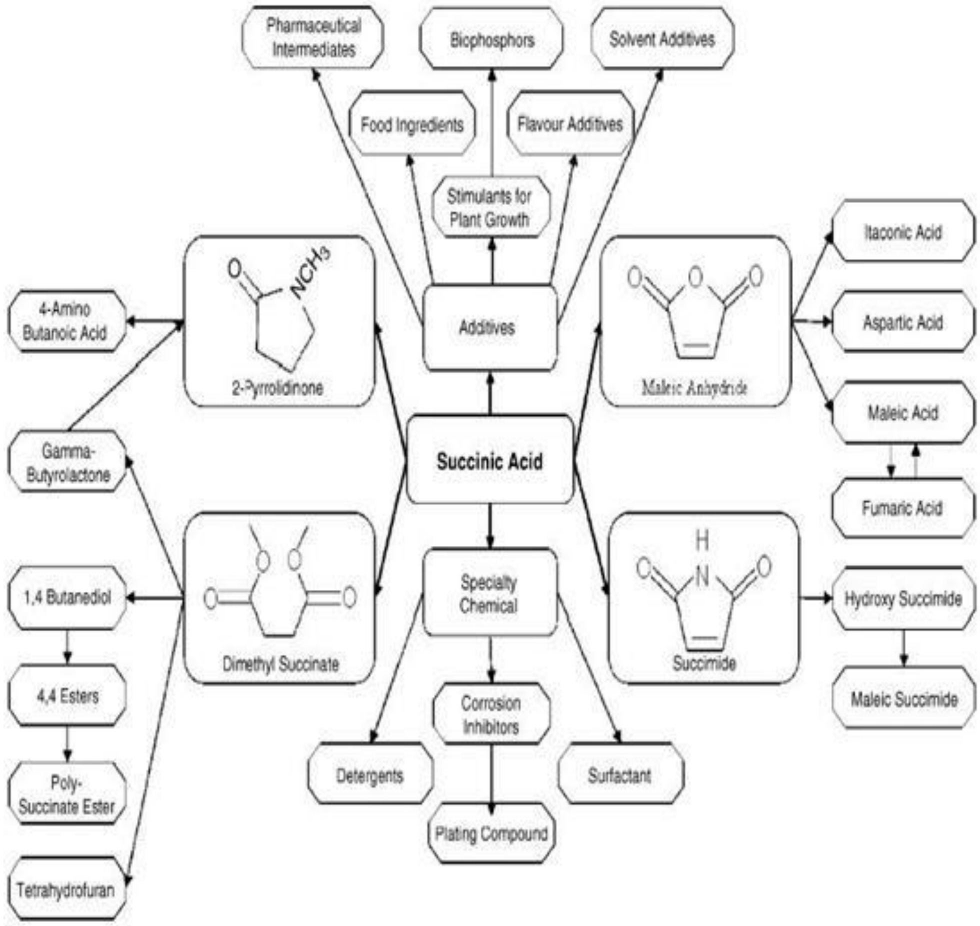

Figure 1.1: Various chemicals and products that can be synthesized from succinic acid

Furthermore, the increasing demand for succinic acid is expected as the uses of succinic acid is extended to the synthesis of glycerol. This is partially due to the high conversion cost of maleic anhydride to succinic acid by the chemical process, which limits the use of succinic acid for the wide range of applications. On the other hand, recent analysis showed that fermentative production of succinic acid from renewable resources can be more cost-effective than the petroleum-based processes (Jain *et al*., 1989).

This research study on the glycerol fermentation in order to produce succinic acid based on substrate concentration and pH. A series of batch fermentations with different initial substrate concentrations and pH were conducted in experiments and the experimental data were used to estimate parameters and also to validate the experiments. In this research also discuss the use of anaerobic fermentation processes for the conversion of glycerol into higher value products. The experiments developed were able to successfully explain the behavior of succinic acid production with times and the fermentative characteristics of succinic acid production from glycerol fermentation process were discussed in detail. The succinic acid produced is measured by using HPLC.

1.2 Problem Statement

Production cost is a major problem for producing succinic acid in order to achieve market acceptance, secure off-take agreements and ensure that plants are operating close to capacity. Production cost of succinic acids by chemical plants is higher compare with production of fermentation process by microorganism. Second problem for chemical plants is large number of unit operations. There are a large number of unit operations such as separation processes, including extraction, absorption, membrane filtration and distillation column. Chemical plants also produce high energy (electricity) because chemical plants are designed on a large scale for continuous operation and provide most of the electrical energy used. Chemical plants highly complex because of multiple generating units. Chemical plants produce toxicity and gives effect to the environment pollution and global warming. The combustion of the plants is discharged to the air and this contains carbon dioxide and water vapor, as well as other substances such as nitrogen, nitrogen oxides, and sulfur oxides. The impact is acid rain and air pollution, and has been connected with global warming. Acid rain is caused by the emission of nitrogen oxides and sulfur dioxide into the air.

1.3 Objectives

1. To study the effect of substrate concentration and pH on succinic acid production.

1.4 Scope of Study

1. To study the effect of substrate concentration at ranges 5 to 25 g/L during glycerol fermentation.
2. To study the effect of pH at ranges 5.5 to 7.5 using sulfuric acids (H_2SO_4) and natrium Carbonat (Na_2CO_3) as a buffer during fermentation process.
3. To determine the amount of succinic acids by using HPLC.

1.5 Rationale and Significance

The microorganism's metabolic pathways are being genetically engineered so they are capable of converting different types of sugars very efficiently. The microorganism can culture in a large amount so the increase the amount of succinic acid production and the impact is expands markets for domestic agriculture and biomass crops. The production of succinic acid through fermentation processes based on sugar feedstocks has the potential to considerably reduce the chemical industry's dependence on fossil-based feedstocks, energy intensive and expensive processes and capital intensive plants. Fermentation process produce less temperature compare with chemical plant with high temperature, high pressure of the equipments. In addition, the possibilities of cost effective succinic acid production by fermentation process is reduce energy or saving the energy. The energy savings from a single combined biological and chemical plant producing chemical components could heat thousands homes for a year. Fermentation process gives the impact of environmental friendly and green friendly because carbon dioxide (CO_2) is consumed during the fermentation process. Fermentation-based production processes and technology are built around microorganisms engineered to produce such chemical products, reducing feedstock, production, plant cost and improving profitability.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Succinic acid, an important four-carbon platform chemical, is mostly being produced by chemical processes using liquefied petroleum gas or petroleum oil as a starting material. Succinic acid produced by various microorganisms can be used as a precursor of many industrially important chemicals in food, chemical and pharmaceutical industries. By using glycerol as a carbon sources the succinic acid can produced with much less by-product formation.

2.2 Background of succinic acid

Succinic Acid is another name for Amber Acid that has been used in Europe as a natural antibiotic and general curative for centuries. Succinic acid a powerful anti oxidant that helps fight toxic free radicals and disruptions of the cardiac rhythm, succinic acid has been shown to stimulate neural system recovery and bolster the immune system, and helps compensate for energy drain in the body and brain, boosting awareness, concentration and reflexes, and reducing stress (Jain *et al*.,1989).

Succinic acid has been reported to be produced and accumulated by anaerobic microorganisms as the major product of their metabolism. Thus, attempts are being made worldwide to screen anaerobic microorganisms for succinic acid production (Zeikus *et al*., 1999).

Succinic Acid (Butanedioic Acid) is a dicarboxylic acid of four carbon atoms. It occurs naturally in plant and animal tissues. It plays a significant role in intermediary metabolism (Krebs cycle) in the body. Krebs cycle (also called citric acid cycle; tricarboxylic acid cycle) is a sequence process of enzymatic reaction which a two-carbon acetyl unit is oxidized to carbon dioxide and water to provide energy in the form of high-energy phosphate bonds. Succinic acid is a colourless crystalline solid with a melting point of 185 to 87 ^{o}C, soluble in water, slightly dissolved in ethanol, ether, acetone and glycerine, not dissolved in benzene, carbon sulfide, carbon tetrachloride and oil ether.

The common method of synthesis of succinic acid is the catalytic hydrogenation of maleic acid or its anhydride. Carboxylic acid can yield acyl halides, anhydrides, esters, amides, and nitriles for the application of drug, agriculture, and food products, and other industrial uses (Zeikus *et al*., 1999).

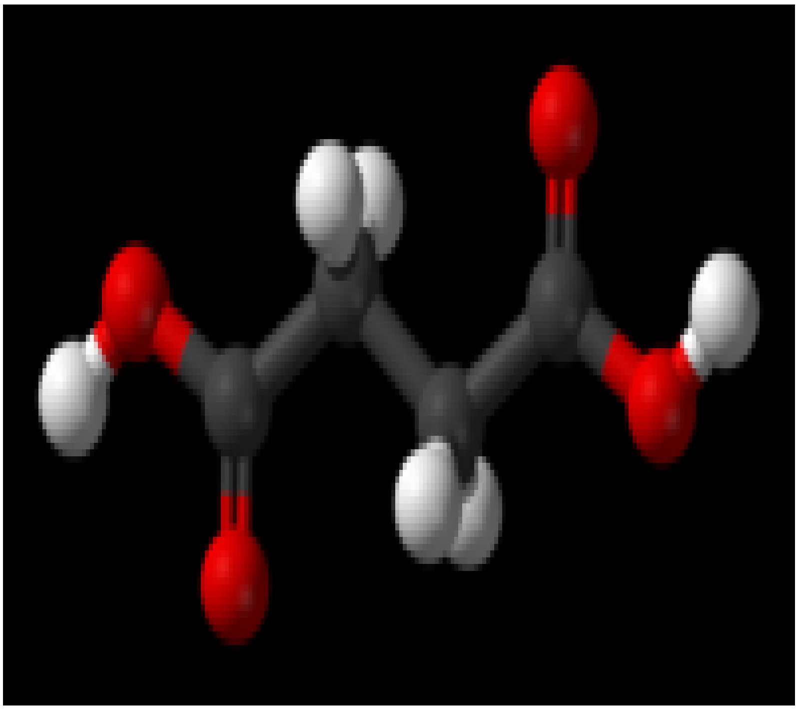

Figure 2.1: Molecule structure of succinic acid

2.3 Production of Succinic acid

2.3.1 Synthesis Process

Currently, the acid is produced commercially through chemical synthesis involving hydrolysis of petroleum products, which is associated with certain environmental hazards.

Maleic acid is converted into maleic anhydride by dehydration, to malic acid by hydration, and succinic acid by hydrogenation (ethanol / Palladium on carbon. It reacts with thionyl chloride or phosphorus pentachloride to give the maleic acid chloride (it is not possible to isolate the mono acid chloride).

Hydrogenation is the chemical reaction that results from the addition of hydrogen (H_2). The process is usually employed to reduce or saturate organic compounds. The process typically constitutes the addition of pairs of hydrogen atoms to a molecule. Catalysts are required for the reaction to be usable, non-catalytic hydrogenation takes place only at very high temperatures. Hydrogen adds to double and triple bonds in hydrocarbon (Podkovyrov and Zeikus, 1993).

Because of the importance of hydrogen, many related reactions have been developed for its use. Most hydrogenations use gaseous hydrogen (H_2), but some involve the alternative sources of hydrogen, not H_2. These processes are called transfer hydrogenations. The reverse reaction, removal of hydrogen from a molecule, is called dehydrogenation. A reaction where bonds are broken while hydrogen is added is called hydrogenolysis, a reaction that may occur to carbon-carbon and carbon-heteroatom (O, N, X) bonds. Hydrogenation differs from protonation or hydride addition: in hydrogenation, the products have the same charge as the reactants. Hydrogenation reaction is the addition of hydrogen to maleic acid to succinic acid.

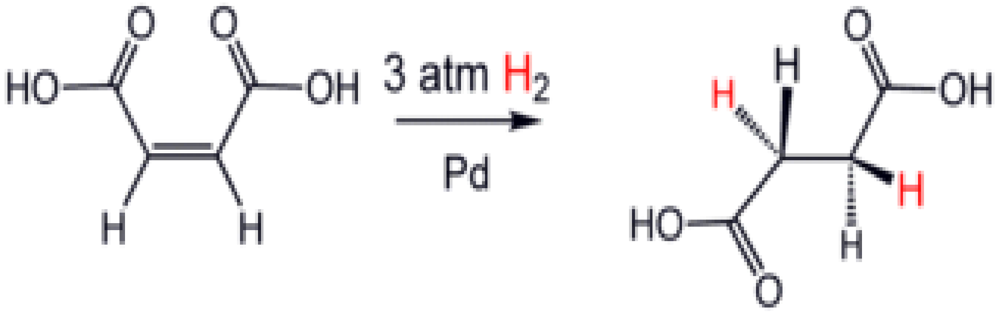

Figure 2.2: The structure for hydrogenation process

2.3.2 Fermentation Process

Fermentation, process by which the living cell is able to obtain energy through the breakdown of glucose and other simple sugar molecules without requiring oxygen. Fermentation is achieved by somewhat different chemical sequences in different species of organisms. Two closely related paths of fermentation predominate for glucose. When muscle tissue receives sufficient oxygen supply, it fully metabolizes its fuel glucose to water and carbon dioxide. However, at times of strenuous activity, muscle tissue uses oxygen faster than the blood can supply it.

During this anaerobic condition, the six-carbon glucose molecule is only partly broken down to two molecules of the three-carbon sugar called lactic acid. This process, called lactic acid fermentation, also occurs in many microorganisms and in the cells of most higher animals. In alcoholic fermentation, such as occurs in brewer's yeast and some bacteria, the production of lactic acid is bypassed, and the glucose molecule is degraded to two molecules of the two-carbon alcohol, ethanol, and to two molecules of carbon dioxide. Many of the enzymes of lactic acid and alcoholic fermentation are identical to the enzymes that bring about the metabolic conversion known as glycolysis. Alcoholic fermentation is a process that was known to antiquity.

Therefore, much attention has been focused in the past few years on fermentative production of succinic acid by anaerobic or facultative anaerobic microorganisms. This is because it is a common intermediate in the metabolic pathway of several anaerobic and facultative anaerobic microorganisms (Chotani *et al.*, 2000). This fermentation process for the production of succinic acid can be regarded as a “green technology” not only because renewable substrates are used for its production but also because CO_2, a green house gas is fixed during microbial production of succinic acid (Lee *et al.*, 2000).

2.4 Factors effect of Fermentation

Fermentation is affected by several factors including the temperature, salt concentration, pH, oxygen availability and nutrient availability. The rate of fermentation can be controlled by manipulating any of these factors (Hong *et al.*, 2003).

Different bacteria tolerate different temperatures. Most have an optimum of between 20 to 30°C although some prefer higher temperatures (50 to 55°C) and others colder (15 to 20°C). Most lactic acid bacteria work best at temperatures of 18 to 22°C. The *Leuconostoc* species which initiate fermentation have an optimum of 18 to 22°C. The *Lactobacillus* species have temperature optima above 22°C. The optimum temperature for pickle fermentation is around 21°C. A variation of just a few degrees from this temperature alters the activity of the microbes and affects the quality of the final product.

Lactic acid bacteria tolerate high salt concentrations, which gives them an advantage over other less tolerant species. This allows the lactic acid fermenters to begin metabolism, which produces acid, which then further inhibits the growth of non-desirable organisms. *Leuconostoc* species tolerate high salt concentrations, which makes them ideal to start the lactic acid fermentation.

Salt plays an important role in initiating the fermentation and also in the quality of the product. The addition of too much salt may inhibit the desirable bacteria and also affect the hardness of the product. The principle function of salt is to withdraw juice from the vegetables and make a favourable environment for fermentation. Salt is generally added to give a final concentration of 2.0 to 2.5%. At this concentration the *Lactobacilli* are slightly inhibited but the *Leuconostoc* are not affected.

The optimum pH for most bacteria is near the neutral point (pH 7.0). Certain bacteria are acid tolerant and will survive at reduced pH levels. Both *Lactobacillus* and *Streptococcus* species are acid tolerant. Some of the fermenters are anaerobes while others require oxygen. Some of the *lactobacilli* are microaerophilic which means they grow in the presence of reduced amounts of oxygen (Samuelov *et al*., 1991).

All bacteria require a source of nutrients for metabolism. The fermenters require carbohydrates, either simple sugars such as glucose and fructose or complex ones such as starch or cellulose. The energy requirements of microbes are very high. Limiting the amount of substrate available can reduce the rate of fermentation.

2.5 Raw material for Glycerol Fermentation

2.51 Glycerol

Glycerol is an organic compound, also called glycerin or glycerine. It is a colorless, odorless, viscous liquid that is widely used in pharmaceutical formulations. Glycerol has three hydrophilic hydroxyl groups that are responsible for its solubility in water and its hygroscopic nature. The glycerol substructure is a central component of many lipids. Glycerol is sweet-tasting and of low toxicity.

Glycerol is an attractive carbon substrate for biological conversion because it is available from renewable resources in large amounts and can be utilized by number of microorganism. Glycerols are produced as a surplus by-product in the growing oleochemical industries for the production soaps, fatty acids, waxes, and surfactants. Therefore, several environmentally friendly processes based on microbial fermentation fermentation have been proposed for glycerol utilization. Some useful chemicals have been produced from glycerol fermentation (Lee *et al*., 2001).

The simplest trihydric alcohol, with the formula $CH_2OHCHOHCH_2OH$. It is widely distributed in nature in the form of its esters, called glycerides. The glycerides are the principal constituents of the class of natural products known as fats and oils. It is completely soluble in water and alcohol but is only slightly soluble in many common solvents, such as ether, ethyl acetate, and dioxane. Glycerin is insoluble in hydrocarbons. It boils at 290°C (554°F) at atmospheric pressure and melts at 17.9°C. Its specific gravity is 1.262 at 25°C (77°F) referred to water at 25°C, and its molecular weight is 92.09. It has a very low mammalian toxicity.

Glycerin is used in nearly every industry. With dibasic acids, such as phthalic acid, it reacts to make the important class of products known as alkyd resins, which are widely used as coating and in paints. It is used in innumerable pharmaceutical and cosmetic preparations; it is an ingredient of many tinctures, elixirs, cough medicines, and anesthetics; and it is a basic medium for toothpaste.

In foods, it is an important moistening agent for baked goods and is added to candies and icings to prevent crystallization. It is used as a solvent and carrier for extracts and flavoring agents and as a solvent for food colors. Many specialized lubrication problems have been solved by using glycerin or glycerin mixtures. Many millions of pounds are used each year to plasticize various materials (Podkovyrov and Zeikus, 1993).

2.52 Yeast

Yeasts are eukaryotic organisms that exist in nature predominantly as single cells. Traditionally, they have played an important role for man, being used for thousands of years in bread-making, brewing, and making certain foods palatable and nutritious. The commercial importance of yeast has grown considerably over the pas few decades, and they are now being used in a variety of fermentative processes for the synthesis of simple sugars and ammonium nitrogen as well as certain fats, vitamins, and proteins. Although there are about 350 recognized species of yeast, only a relatively small number are important commercially, notably members of the genera *saccharomyces* and *candida*.

Species of *saccharomyces*, such as *S.cerevisiae* and *S.uvarum* (which is sometimes referred to as *S.carlsbergensis*) are used in the manufacture of breads, wines and beers, while some species of Candida are sources of animal food and fodder. One species in particular, *S.cerevisiae,* has received and enormous amount of attention from generations of biologists. This is not so much due to its commercial importance, but rather because it is amenable to most of the cultural techniques and genetic manipulations used with laboratory bacteria. It has been extremely well-characterized genetically and defined mutations in most biochemical pathways have been established.

The genetic engineering of yeast using recombinant DNA techniques has been made possible by the development, recently, of a transformation system and the construction of DNA cloning vectors which mediate the introduction of DNA into yeast cells. These technological advances, combined with classical genetic studies, have provided a wealth of knowledge of yeast molecular biology. The ability to introduce individual genes into yeast and apply selection by complementation to identify a clone carrying a particular gene now permits the isolation of virtually any yeast gene provided that a suitable recipient yeast strain is available. Furthermore, gene cloning in yeast is likely to be of great industrial importance, not only for improvement of existing strains used in fermentation processes, but also for the bulk production of commercially important proteins not selectable in or compatible with bacterial systems (Swarna and Rao.,1993)

2.53 Trypton

Tryptone is the assortment of peptides formed by the digestion of casein by the protease trypsin. Tryptone is commonly used in microbiology to produce Lysogeny broth for the growth of *E. coli* and other microorganisms. It provides a source of amino acids for the growing bacteria. Tryptone is similar to casamino acids, both being digests of casein, but casamino acids can be produced by acid hydrolysis and typically only have free amino acids and few peptide chains

2.6 Anaerobic Fermentation

Under anaerobic conditions, the pyruvate molecule can follow other anaerobic pathways to regenerate the NAD^+ necessary for glycolysis to continue. These include alcoholic fermentation and lactate fermentation. In the absence of oxygen the further reduction or addition of hydrogen ions and electrons to the pyruvate molecules that were produced during glycolysis is termed fermentation.

This process recycles the reduced NADH to the free NAD^+ coenzyme which once again serves as the hydrogen acceptor enabling glycolysis to continue. Alcoholic fermentation, characteristic of some plants and many microorganisms, yields alcohol and carbon dioxide as its products. Yeast is used by the biotechnology industries to generate carbon dioxide gas necessary for bread-making and in the fermentation of hops and grapes to produce alcoholic beverages. Depending on the yeast variety used, the different alcohol levels realized act as a form of population control by serving as the toxic element which kills the producers. Birds have been noted to fly erratically after they have gorged themselves on the fermenting fruit of the Pyracantha shrub (Macy *et al*., 1978).

Reduction of pyruvate by NADH to release the NAD^+ necessary for the glycolytic pathway can also result in lactate fermentation, which takes place in some animal tissues and in some microorganisms. Lactic acid-producing bacterial cells are responsible for the souring of milk and production of yogurt. In working animal muscle cells, lactate fermentation follows the exhaustion of the ATP stores. Fast twitch muscle fibers store little energy and rely on quick spurts of anaerobic activity, but the lactic acid that accumulates within the cells eventually leads to muscle fatigue and cramp (Hong *et al*., 2003).

Many anaerobic and facultative anaerobic microorganisms ferment carbohydrates to a mixture of acids, e.g. formate, acetate, lactate and succinate as end products. Phosphoenol pyruvate (PEP) is one of the central intermediates during the mixed acid fermentation. It is either converted into pyruvate resulting in the formation of the fermentation products acetate, formate, ethanol and lactate or it is converted into oxaloacetate resulting in the formation of end products succinate and propionate via the reversible arm of tricarboxylic acid (TCA) cycle (Clark, 1989).

Under anoxic conditions, the flux of PEP towards either oxaloacetate or puruvate is affected by environmental factors such as pH, temperature, hydrogen, carbon dioxide and nutritional factors as carbon, nitrogen sources and metal ions of the growth medium.